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Failure to Phosphorylate the Retinoblastoma Gene Product in Senescent Human Fibroblasts

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Heterokaryon studies suggest that senescent and quiescent human diploid fibroblasts (HDF) contain a common inhibitor of entry into S phase. DNA synthesis can be induced in senescent and quiescent HDF by fusing them with cells containing DNA viral oncogenes such as SV40 T antigen, adenovirus E1A, or human papillomavirus E7. Both senescent and quiescent HDF contained the unphosphorylated form ($p110^{Rb}$) of the retinoblastoma protein, a putative inhibitor of proliferation. After serum stimulation, senescent HDF did not phosphorylate $p110^{Rb}$ and did not enter S phase, whereas quiescent HDF phosphorylated $p110^{Rb}$ and entered S phase. These findings, combined with the observations that T antigen, E1A, and E7 form complexes with, and presumably inactivate, unphosphorylated $p110^{Rb}$, suggest that failure to phosphorylate $p110^{Rb}$ may be an immediate cause of failure to enter S phase in senescent HDF.

HUMAN DIPLOID FIBROBLASTS (HDF) have a finite proliferative life-span at the end of which the cells remain alive in a G_1-arrested senescent state (*1*). Previous studies of the interaction of senescent HDF and replicating HDF in heterodikaryons have suggested that senescent HDF contain an inhibitor of entry into S phase because (i) they cannot be induced to synthesize DNA by fusion to replicating HDF and (ii) entry into S phase was inhibited in the replicative nuclei in these heterokaryons (*2*). Carcinogen-transformed HDF and several human tumor-derived cell lines were similarly inhibited from initiating DNA synthesis in heterodikaryons formed with senescent HDF (*3*). However, SV40-transformed HDF, adenovirus 5 early region–transfected human kidney cells (line 293), and HeLa cells, which express human papillomavirus (HPV) 18 DNA sequences (*4*), were not sensitive to inhibition by senescent HDF; rather, they were able to induce DNA synthesis in the senescent nuclei in heterodikaryons (*3, 5*). When young HDF were made quiescent by either serum deprivation or high cell density, they exhibited a similar inhibitory activity in the heterokaryon assay and were also induced to synthesize DNA by fusion with SV40-transformed HDF, 293 cells, and HeLa cells (*6, 7*). These data suggest the possibility (*3, 6*) that a common inhibitor was operating in senescent and quiescent cells and that cells transformed by certain DNA tumor viruses have a transforming factor that can override the putative inhibitor of DNA synthesis. Is the hypothesis of a common inhibitor consistent with the fact that the inhibition is reversible by serum stimulation in quiescent HDF but not in senescent HDF? This could occur because of changes in the ability of the senescent cells to respond to mitogens such that they are functionally mitogen-deprived even when they are fed with fresh serum.

SV40 T antigen, adenovirus E1A, and HPV E7 each can form a complex with the retinoblastoma susceptibility gene product (RB) (*8–10*). RB is considered to be an inhibitor of cell proliferation on the basis of its absence or inactivation in retinoblastoma and a subset of other human tumors and the ability of the wild-type gene (*RB1*) to suppress tumorigenicity when reintroduced into retinoblastoma and osteosarcoma cells (*11*). RB, which is unphosphorylated in G_0 or G_1 phase cells, becomes phosphorylated at the G_1/S boundary, suggesting that phosphorylation may be necessary for entry into S phase (*12–15*). As SV40 T antigen binds only the unphosphorylated form of RB (*16*), a simple hypothesis is that unphosphorylated RB inhibits entry into S phase and that inhibition can be relieved by either phosphorylation or binding to one of the three aforementioned viral oncogenes.

As an initial step in our study of the role of RB in cellular senescence, we investigated whether or not *RB1* is overexpressed in senescent HDF. Northern blots containing equal amounts of polyadenylated RNA from replicating, quiescent, and senescent HDF were analyzed for expression of *RB1* (*17*) and the cytoplasmic β-actin gene (*18*). The intensity of the RB band relative to the band representing β-actin was ~35% less for senescent HDF than for replicating HDF or quiescent HDF. This comparison may underestimate the difference in *RB1* expression because a recent report (*19*) suggests that β-actin expression in senescent HDF is about half of that in replicating HDF or quiescent HDF. In any case, our data clearly show that *RB1* is not overexpressed in senescent HDF.

The synthesis of RB in senescent HDF was examined by immunoprecipitation of ^{35}S-labeled proteins with the C36 monoclonal antibody (*8*) to human RB (Fig. 1A). The senescent cells, which had failed to achieve even one population doubling (PD) in 3 weeks with weekly refeeding, had predominantly the unphosphorylated main form of RB ($p110^{Rb}$). There was little or no phosphorylated main band, which can be seen as $pp112–116^{Rb}$ in the replicating cell profile. In addition, there was no incorporation of ^{32}P in this region in immunoprecipitates prepared from senescent HDF metabolically labeled with [^{32}P]orthophosphate (*20*). In other studies, phosphorylation of the 112- to 116-kD bands in replicating cells has been shown by metabolic labeling with [^{32}P]orthophosphate and by sensitivity to alkaline or acid phosphatase (*12, 14–16*). The phosphorylated character of these bands in replicating IMR-90 was confirmed by showing that they were sensitive to digestion with alkaline phosphatase (Fig. 1B).

In a few studies, additional lower molecular weight forms of RB ($p98^{Rb}$ and $pp100–104^{Rb}$) have been described that were attributed to translation of RB beginning at a

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second in-frame AUG codon (*14, 15, 21, 22*). There is some material in this molecular weight region in both the senescent cells and replicating cells in Fig. 1A. The amount of this material in senescent cells was somewhat variable but never very large (for example, Figs. 1A and 3).

When senescent HDF are stimulated with fresh serum-containing medium, they undergo many of the same prereplicative events as do quiescent HDF that are stimulated to enter S phase. For example, they increase their expression of 11 cell cycle–regulated genes including c-*myc*, c-H-*ras*, and histone H3, and they increase their levels of enzymes involved in DNA synthesis such as thymidine kinase and DNA polymerase α (*23*). However, they do not enter S phase. To investigate whether phosphorylation of RB occurs in serum-stimulated senescent HDF, we first determined the time course of RB phosphorylation in serum-stimulated quiescent HDF. RB was phosphorylated at 15 to 18, 18 to 21, and 21 to 24 hours after stimulation of quiescent IMR-90 cells (Fig. 2). The effect of serum stimulation on DNA synthesis in quiescent IMR-90 cells was measured by labeling the cells with [^{3}H]thymidine for either 24 hours before stimulation (3% labeled nuclei) or for 15, 18, and 21 hours after stimulation (12, 36, and 42% labeled nuclei, respectively). Taken together, these data are consistent with the hypothesis that phosphorylation of RB begins at the G_1/S boundary. Both the predominant form and lower molecular weight form of RB are phosphorylated in these cells. The predominance of phosphorylation of the lower molecular weight band was a reproducible effect in two independent experiments with ^{35}S-labeled serum-stimulated quiescent IMR-90. However, when steady-state levels of RB were examined in serum-stimulated quiescent cells, there was only a small amount of lower molecular weight form (for example, Fig. 3). This suggests that the phosphorylated lower molecular weight form may be relatively short lived. The physiological significance of this observation is not known.

When senescent HDF were labeled with [^{35}S]methionine at 18 to 21 hours (Fig. 4) and 21 to 24 hours (*20*) after serum stimulation, there was no apparent phosphorylation of RB. This was true whether or not the senescent cells were deprived of serum before stimulation (Fig. 4) (*20*). In addition, a portion of the immunoprecipitated RB in Fig. 4 was treated with alkaline phosphatase to test further whether any of the bands were phosphorylated, even though they exhibited the mobility of unphosphorylated RB. There was no apparent difference between the treated and untreated samples. In these experiments, the [^{3}H]thymidine labeling index of the senescent HDF was also measured over a 24-hour period both before and after stimulation. The unstimulated cells had an average of 0.07% labeled nuclei (range 0.01 to 0.2%), and the stimulated cells had an average of 1.32% labeled nuclei (range 0.02 to 4%), which indicates that there was no significant induction of DNA synthesis in these cells. Taken together, these data suggest that at least one reason that senescent HDF fail to enter S phase could be because they fail to phosphorylate their RB.

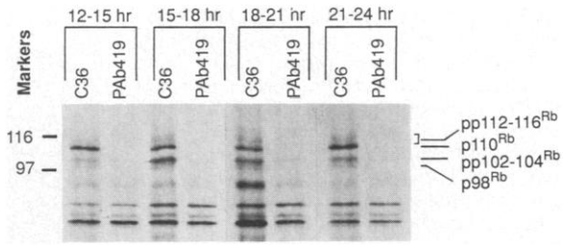

Fig. 2. Phosphorylation of RB after serum stimulation of quiescent HDF. Young IMR-90 cells (PD 29) were grown to quiescence after 2 weeks in medium plus 10% FBS. At that time, they had a [^{3}H]thymidine labeling index of 3% in 24 hours. The time course of phosphorylation of RB was examined by labeling equal numbers of cells with [^{35}S]methionine during the intervals 12 to 15, 15 to 18, 18 to 21, and 21 to 24 hours after serum stimulation. Immunoprecipitations and analysis were as described in the legend to Fig. 1A.

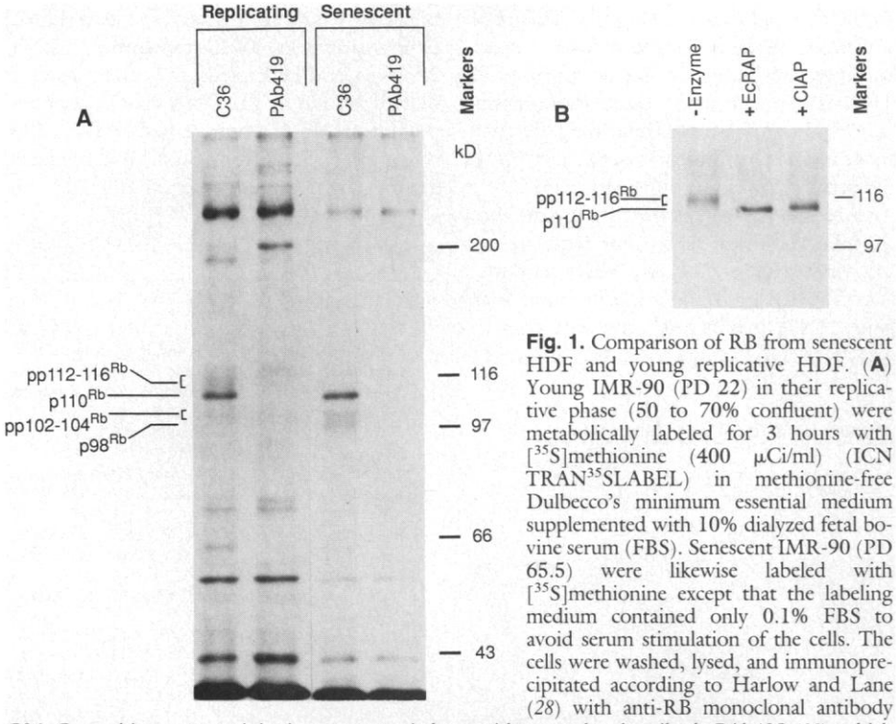

Fig. 1. Comparison of RB from senescent HDF and young replicative HDF. (**A**) Young IMR-90 (PD 22) in their replicative phase (50 to 70% confluent) were metabolically labeled for 3 hours with [^{35}S]methionine (400 μCi/ml) (ICN TRAN^{35}SLABEL) in methionine-free Dulbecco's minimum essential medium supplemented with 10% dialyzed fetal bovine serum (FBS). Senescent IMR-90 (PD 65.5) were likewise labeled with [^{35}S]methionine except that the labeling medium contained only 0.1% FBS to avoid serum stimulation of the cells. The cells were washed, lysed, and immunoprecipitated according to Harlow and Lane (*28*) with anti-RB monoclonal antibody C36. Control immunoprecipitations were carried out with monoclonal antibody PAb419 (*29*), which recognizes SV40 T antigen, an antigen that is not present in normal HDF. The immunoprecipitates and molecular weight markers (Bio-Rad) were fractionated on 7% SDS-polyacrylamide gels, stained with Coomassie blue, prepared for fluorography, and exposed to Kodak X-AR film for 0.5 days (replicative HDF) and 10 days (senescent HDF) at −70°C to normalize the amount of RB seen in the two types of cells. The different exposure times reflect the fact that the senescent cell lysate had incorporated <10% as much radioactivity as had the replicating cell lysate. (**B**) Dephosphorylation of RB by treatment with alkaline phosphatase. RB was immunoprecipitated from unlabeled replicating IMR-90, divided into three equal parts, resuspended in an alkaline buffer (100 mM NaCl, 5 mM $MgCl_2$, and 100 mM tris-HCl, pH 8.0) and incubated at 37°C with (i) no enzyme for 1 hour, (ii) 6 units of *Escherichia coli* recombinant alkaline phosphatase (EcRAP) (Pharmacia) for 15 min, or (iii) 96 units of calf intestine alkaline phosphatase (CIAP) (Boehringer Mannheim Biochemical) for 1 hour. The samples were run on gels as above, transferred to nitrocellulose, and immunoblots were prepared with antibody PMG3-245 (2 μg/ml) (Pharmingen), which is specific for human RB. A 1:500 dilution of alkaline phosphatase conjugated rabbit antibody to mouse immunoglobulin G1 (IgG1) (Pharmingen) was used as the secondary antibody, and color was developed in 100 ml of tris-Mg buffer (100 mM tris, pH 9.5, and 0.5 mM $MgCl_2$) containing 30 mg of 4-nitro blue tetrazolium chloride and 15 mg of 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim Biochemical). In a repeat of this experiment, dephosphorylation of the 112- to 116-kD bands was also seen with half as much CIAP and four times as much EcRAP.

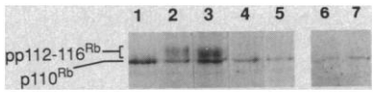

Fig. 3. Proportion of total RB that is phosphorylated in senescent, quiescent, and replicating HDF. Whole-cell lysates were prepared from unstimulated quiescent HDF (lane 1); quiescent HDF 24 hours after stimulation (lane 2); replicating HDF (lane 3); unstimulated senescent HDF (lane 4); senescent HDF 24 hours after stimulation (lane 5); unstimulated senescent HDF (lane 6); and senescent HDF 30 hours after stimulation (lane 7). Cells that had been washed with phosphate-buffered saline (PBS) were harvested by scraping, washed again with PBS, and resuspended in 100 μl of lysis buffer (*28*). Following 30 min of lysis at 4°C, the lysates were clarified at 15,000*g* for 15 min at 4°C. Aliquots containing 140 μg of protein from each lysate were analyzed by immunoblotting as described in the legend to Fig. 1B.

An alternative explanation for the preceding data is that phosphorylation of RB was not seen in serum-stimulated senescent HDF because it occurred after the 18- to 24-hour window of time that was examined. We think that this is not true for the following reasons. First, when old quiescent HDF (a few PD before senescence) are stimulated with serum, those cells that enter S phase do so only a little more slowly (1.5- to 3-hour delay) than do young quiescent HDF (*24*). These data and those on gene expression and enzyme activity after serum stimulation (*23*) suggest that the time course of many G_0 to S phase events is not drastically altered in senescent or nearly senescent cells. Second, we used immunoblotting to examine the phosphorylation status of total RB, rather than just newly synthesized RB, in senescent HDF at 24 and 30 hours after stimulation. These data (Fig. 3) show that there was no significant phosphorylation of RB in senescent HDF as late as 30 hours after stimulation. Because the immunoblotting experiments were done with a different monoclonal antibody (PMG3-245) than were the immunoprecipitation experiments (C36), they also argue against the possibility that the binding or accessibility of a single monoclonal antibody to the phosphorylated forms of RB was somehow different in senescent cells compared to young cells. Finally, the immunoblotting data show a higher proportion of phosphorylated RB in replicating HDF and stimulated quiescent HDF than do the metabolic labeling studies. This phenomenon, which is consistent with a delay in phosphorylation of newly synthesized RB, has been noted in other experiments (*12, 15*). It emphasizes further the difference between serum-stimulated senescent HDF and serum-stimulated quiescent HDF.

Since many studies now suggest that phosphorylation of RB is a prerequisite for entry into S phase (*12–16*), failure to phosphorylate RB could be a specific molecular cause of the inability of senescent cells to synthesize DNA. Likewise, the ability of SV40-transformed HDF, 293 cells, and HeLa cells to bind RB could account for their ability to induce DNA synthesis in senescent and quiescent HDF. However, it is possible that other functions are also required because microinjection of the E1A gene (+c-H-*ras*) into senescent HDF did not induce DNA synthesis (*25*). In heterokaryons where DNA synthesis is inhibited, our hypothesis is that RB contributed by senescent HDF or quiescent HDF remains unphosphorylated. If so, then one must ask why the replicating cells, which are capable of phosphorylating their own RB when they are unfused, cannot phosphorylate RB contributed by a senescent or quiescent cell in a heterodikaryon. Several possibilities are (i) a stoichiometric effect, (ii) a phosphatase that acts on RB, or (iii) an inhibitor of RB phosphorylation. Alternatively, there could be another inhibitor of DNA synthesis in senescent and quiescent HDF. Since such an inhibitor would also have to be overridden or inactivated by SV40-transformed HDF, 293 cells, and HeLa cells, the other proteins that are bound by the DNA viral oncogenes present in SV40-transformed HDF, 293 cells, and HeLa cells would be candidates for this last possibility (*22, 26, 27*).

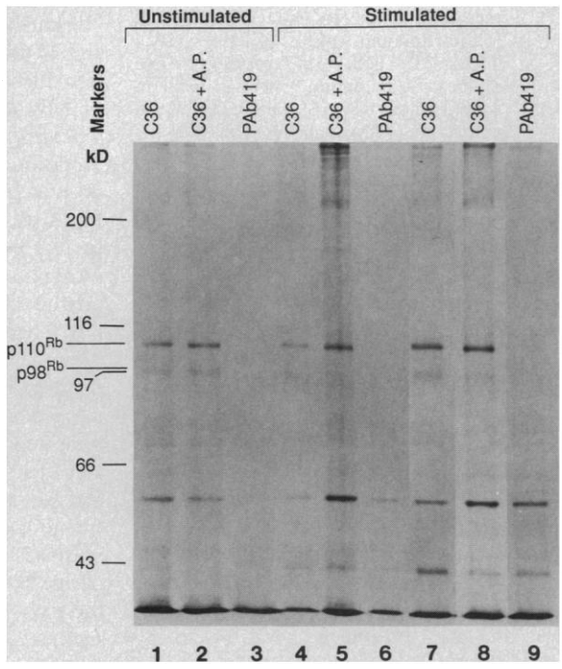

Fig. 4. Effect of serum stimulation on phosphorylation of RB in senescent HDF. IMR-90 cells were grown to senescence (PD 66.5) in medium containing 10% FBS and then incubated in medium containing 0.1% FBS for 4 days before serum stimulation. Half of the cells were refed with fresh medium containing 10% FBS for 18 hours and then incubated in methionine-free medium containing 10% dialyzed FBS and [^{35}S]methionine (400 μCi/ml) for 3 hours. The unstimulated cells were labeled in the same way except that the labeling medium contained only 0.1% FBS. The cells were harvested, immunoprecipitated with C36 or PAb419, and analyzed as described in the legend to Fig. 1A. In addition, aliquots of the C36-immunoprecipitated proteins were treated with 24 units of CIAP as described in the legend to Fig. 1B. Approximately equal numbers of ^{35}S counts per minute were loaded in lanes 1 to 6, even though the stimulated cells incorporated approximately seven times as much label per cell as did the unstimulated cells. Half of the remaining stimulated cell samples were loaded in lanes 7 to 9 to provide additional evidence that there is little or no phosphorylated RB in serum-stimulated senescent HDF.

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Restoration of the Plasticity of Binocular Maps by NMDA After the Critical Period in *Xenopus*

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Visual input during a critical period of development plays a major role in the establishment of orderly connections in the developing visual system. In *Xenopus laevis*, the matching of visual maps from the two eyes to the optic tectum depends on binocular visual input during the critical period, which extends from late tadpole to early juvenile stages. Alterations in eye position, which produce a mismatch of the tectal maps, normally evoke a compensatory adjustment in the map of the ipsilateral eye only during the critical period. However, continuous application of the glutamate receptor agonist *N*-methyl-D-aspartate (NMDA) after the normal end of the critical period restores this ability to realign the visual map.

VISUAL EXPERIENCE CAN HAVE A major impact on the developing axonal connections of the visual system. In particular, factors such as imbalance or misalignment of input from the two eyes alter the axonal connections that underlie binocular maps. The period during which a set of connections is modifiable is referred to as the critical period for that projection (*1*). The cellular events that normally lead to loss of plasticity during maturation are still unknown. During the critical period, application of drugs that interfere with synaptic function can interfere with plasticity. For example, blockage of NMDA-type glutamate receptors prevents visually evoked reorganization of ocular dominance columns in kitten visual cortex (*2*) and of binocular maps in the tectum of juvenile *Xenopus laevis* (*3*). These results have led us to consider whether normal loss of plasticity reflects a decrease in NMDA receptor function and to test whether application of NMDA can reverse the normal loss of plasticity in *Xenopus* tectum.

In *Xenopus*, each tectal lobe receives binocular input. The projection of the contralateral eye is relayed directly by way of the optic nerve, whereas the projection of the ipsilateral eye is relayed indirectly, by way of the opposite tectal lobe and the nucleus isthmi (*4*) (Fig. 1). The two projections form topographic maps of the visual field in the tectum that are in register with each other. The map for the ipsilateral eye comes into alignment with the map for the contralateral eye by active adjustment of axonal connections during development (*5*), when the ipsilateral projection displays dramatic plasticity (*6*). For example, if one eye is rotated by 90° in a midlarval tadpole, while the other eye remains in its normal orientation, the maps of the two eyes initially are misaligned by 90°. However, over the next 2 to 3 months, the ipsilateral map on each tectal lobe becomes reoriented to match the rotated contralateral map (*7*). This realignment normally occurs only during the critical period of development, which ends at about 3 months after metamorphosis. As the frog ages, the ipsilateral map loses the capacity to reorient in response to eye rotation (*8*).

What processes bring these two maps into register? Visually elicited neuronal activity appears to be crucial, since alignment of the ipsilateral map to match the contralateral map requires binocular visual input during development (*5*). One mechanism proposed for activity-dependent refinement of retinotectal maps in frogs and fish (*9*) involves selective stabilization of those retinotectal synapses located on tectal cells receiving input from other retinotectal axons that fire in a correlated pattern. Such correlation is most likely to occur for axons with overlapping receptive fields. The stabilization process seems to be triggered by activation of the NMDA-type glutamate receptor (*10, 11*). Sufficient activity would depolarize tectal cell dendrites by non–NMDA-type glutamate receptors, and the depolarization would expel Mg^{2+} ions from the NMDA receptor channel, allowing influx of Na^+ and Ca^{2+} (*12*). The Ca^{2+} could induce some transient change in the dendrite, allowing it to stabilize recently active retinotectal synapses. We propose a comparable mechanism for activity-dependent matching of the ipsilateral (isthmotectal) and contralateral (retinotectal) maps in *Xenopus*. When isthmotectal and retinotectal axons with corresponding receptive fields converge on a given tectal cell, the NMDA-dependent mechanism would stabilize the isthmotectal synapses (Fig. 2). Consistent with this proposed role for NMDA receptors, we have shown that in *Xenopus*, blocking the NMDA receptor during the critical period of development prevents the ipsilateral map from

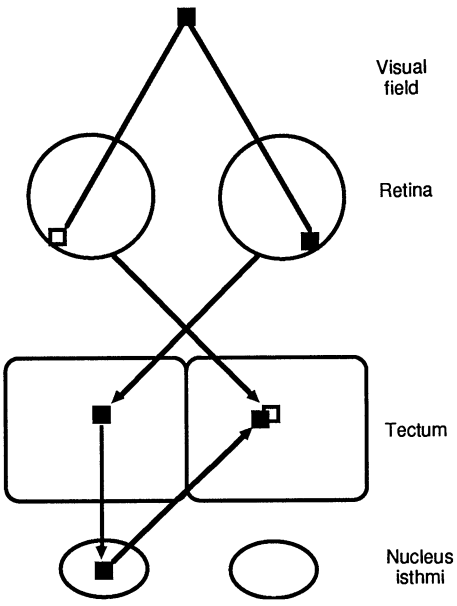

Fig. 1. Neural connections from the eyes to the optic tectum. The right lobe of the tectum receives input from the left eye directly by way of the retinotectal projection and from the right eye indirectly by way of the left nucleus isthmi.

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